2475-Pos Board B245
Dynamic Switch Between Assembly States of the Human Bloom's Syndrome Helicase during Homologous Recombination
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During normal cellular functioning, various endogenous and exogenous DNA-damaging effects arise, including the production of reactive oxygen species and UV irradiation. Double-stranded DNA breaks (DSB) represent one of the most toxic forms of DNA damage, which can be lethal for the cell or induce malignant transformation. Homologous recombination (HR) is an essential and evolutionarily conserved pathway for the error-free repair of DSBs. However, excessive HR can also lead to harmful large-scale genome rearrangements. HR events are therefore tightly regulated. A key player in HR is the human Bloom's syndrome helicase (BLM). During HR, BLM exerts various molecular activities. Based on ensemble biochemical and single-molecule AFM studies we show that the different actions of BLM during HR take place in different oligomeric forms of the enzyme. During single-stranded DNA translocation, which serves as a basis for quality control of HR via disruption of Rad51 nucleoprotein filaments, BLM functions as a monomer. Contrary, more complex DNA structures resembling later HR intermediates, including D-loops and Holliday junctions, induce dimerization and higher-order oligomerization of BLM. The results indicate that BLM exists in a dynamic equilibrium between different assembly states, which is modulated by the structure of DNA intermediates encountered during HR.

2476-Pos Board B246
Single-Molecule Studies of Restriction Endonuclease Kinetics
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Under optimal conditions, restriction endonucleases are capable of mediating remarkably specific DNA cleavage. This quality makes the restriction endonuclease an indispensible tool for genetic modification and manipulation. However, the mechanism by which restriction endonucleases effectively discriminate between their cognate site and other DNA sequences is not fully understood. Under certain conditions, many restriction endonucleases display ''star activity'' - relaxed specificity resulting in DNA cleavage at sequences that differ from their normal recognition sequence - but the mechanism by which specificity is relaxed is not fully understood. Although at least 600 of the almost 4000 restriction endonucleases that have been identified are commercially available in purified form, DNA cleavage kinetics of only a few of these enzymes have been studied in detail. We have developed a fluorescence-based approach with which we can track the progress of the cleavage reaction in real time, and simultaneously determine the values of the kinetic constants for a particular restriction endonuclease at a specific sequence. Modeling restriction endonuclease-mediated DNA cleavage as a Michaelis-Menten-like process, we expected reaction rates to display a hyperbolic dependence on substrate concentration, but our measurements deviate from this dependence, especially under conditions associated with increased star activity (such as low ionic strength). These observations suggest that substrate inhibition may be a part of the reaction mechanism under normal conditions, and that star activity may be a result of an increase in the population of this pathway. Using high density arrays of femtoliter-sized reaction vessels created by selectively etching bundled optical fibers, we can observe the cleavage activity of hundreds of individual restriction endonuclease molecules in solution. By characterizing the population distribution of single-enzyme turnover rates under a variety of conditions, we hope to gain insight into the reaction mechanisms of both specific cleavage and star activity.

2477-Pos Board B247
G4 Quadruplex Recognition in the Human DEAH-Box Helicase RHAU
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G4 quadruplexes of nucleic acids are very stable stacks of four guanylates that are associated in co-planar fashion via hydrogen bonds. Monovalent cations such as potassium or sodium are bound in the center of the quartet of guanyl groups. G4 quadruplexes can only be unwound enzymatically in vivo and this requires an energy source like ATP. G4 quadruplexes provide therefore the means to regulate nucleic acid specific cellular processes such as telomerase activity or gene expression. The recently discovered DEAH-box helicase RHAU, also known as DHX36, binds to G4 DNA and RNA quadruplexes and unwinds them. G4 quadruplex recognition occurs in a short stretch of amino acids in the N-terminal domain of RHAU, named ''RHAU specific motif''. Using a combination of biophysical methods such as Circular Dichroism, Dynamic Light Scattering, Size Exclusion Chromatography, Analytical Ultracentrifugation, Small Angle X-ray Scattering, Nuclear Magnetic Resonance and X-ray crystallography we are striving to shed light into how RHAU recognizes and binds G4 DNA and RNA complexes.

2478-Pos Board B248
Single Molecule Observation of MicroRNA Processing Enzymes at Action
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MicroRNAs (miRNAs) regulate mRNA expression via RNA interference. MicroRNA is generated when nuclear RNase III Drosha cleaves a long primary transcript liberating a hairpin precursor miRNA (pre-miRNA). The pre-miRNA is processed into a ~22 nt mature miRNA by cytoplasmic RNase III Dicer. In stem cells and certain cancer cells, the maturation process is interrupted when the pre-miRNA is hijacked by Lin28, a pluripotency factor, and the 3' end of the RNA is uridylated by a noncanonical polyA polymerase, TUT4.
With single molecule fluorescence microscopy, here we reveal the action mechanisms of human Dicer and TUT4 proteins at the molecular level. As it is difficult to prepare as large eukaryotic proteins as human Dicer and TUT4 proteins, we used a single molecule immunoprecipitation technique that we had recently developed [1].
We, for the first time, observed Dicer's cleavage activity in real time using single molecule FRET and revealed stepwise processes of the cleavage action. Next, we investigated how Lin28 interfered with Dicer's activity and mediated TUT4's uridylation of pre-miRNA. Our real-time analysis suggests that Lin28 functions as a processivity factor of TUT4 and TUT4 utilizes a looping mechanism when it attaches uridines to a recruited pre-miRNA.
Reference:
[1] K. H. Yeom, I. Heo, J. Lee, S. Hohng, V. N. Kim, C. Joo, (2011) ''Single Molecule Approach to Immunoprecipitated Protein Complexes: Insights into MiRNA Uridylation'' EMBO Reports, 12, 690-696.

2479-Pos Board B249
Helicase-Initiated Assembly of the Primosome
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A direct quantitative analysis of the initial steps in primosome assembly, involving PriA and PriB proteins and the minimal primosome assembly site (PAS) of phage ϕX174, has been performed using fluorescence intensity, fluorescence anisotropy titration, and fluorescence resonance energy transfer techniques. We show that two PriA molecules bind to the PAS at both strong and weak binding sites on the PAS structure without detectable cooperative interactions. Binding of the PriB dimer to the PriA - PAS complex dramatically increases PriA's affinity for the strong site, but only slightly affects its affinity for the weak site. Interactions are driven by apparent entropy changes, with binding to the strong site accompanied by a large unfavorable enthalpy change. The PriA - PriB complex, formed independently of the DNA, is able to directly recognize the PAS structure. Thus, the high-affinity state of PriA for PAS is generated through PriA - PriB interactions. The effect of PriB is specific for PriA-PAS association, but not for PriA-double-stranded DNA or PriA-single-stranded DNA interactions. Only complexes containing two PriA molecules can generate a profound change in the PAS structure in the presence of ATP. The obtained results provide a quantitative framework for initiation of primosome formation and elucidation of further steps in the assembly process.

2480-Pos Board B250
Non-Equilibrium Topology Simplification by Type II Topoisomerases: A Test of Kinetic Proofreading
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Type II topoisomerases are essential enzymes that modify DNA topology. These topoisomerases pass a double stranded segment (T-segment) of DNA though a transient double stranded break in a second segment (G-segment).

This strand passage mechanism allows type II topoisomerases to unlink, unknot, and relax supercoiled DNA to below equilibrium levels[1]. The mechanisms underlying this non-equilibrium topology simplification remain speculative. Several theoretical models have been proposed but experimental data has not been able to distinguish among them. One model developed by Yan and Marko[2], postulates that the strand passage mechanism of type II topoisomerases is governed by a kinetic proofreading process. In practice, this model suggests that type II topoisomerases require two collisions between the T- and G-segments prior to strand passage. The first collision of a T-segment with the enzyme-bound G-segment transiently activates the enzyme and the second collision of the T-segment with the activated enzyme results in strand passage. The model predicts that the strand passage probability scales as the square of the collision rate, which has not been tested. We directly tested this prediction of the kinetic proofreading model using a single-crossing DNA unlinking assay. We measured the rate that topoisomerase IV, a bacterial type II topoisomerase, unlinked DNA as a function of the strand collision probability obtained from Monte Carlo simulations of the DNA crossings. The unlinking rate was linearly related to the collision probability, which is inconsistent with the kinetic proofreading model.

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2.Yan, J., Magnasco, M. O. & Marko, J. F. A kinetic proofreading mechanism for disentanglement of DNA by topoisomerases. *Nature* **401**, 932-935, doi:10.1038/44872 (1999).

2481-Pos Board B251

An Enthalpic "On/Off Switch" Linking DNA Binding and Nucleotide Incorporation Activity in Pol I DNA Polymerases

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Primer-template DNA (pt-DNA) binding by DNA polymerases is the first step of the polymerization cycle. We have previously characterized the thermodynamics of pt-DNA binding with respect to temperature for the Klenow and Klentaq ''large fragment domains'' of DNA polymerase I, from *Thermus aquaticus* and *Escherichia coli*, respectively. DNA binding affinities for both polymerases are quite tight across wide temperature ranges, 5-70 °C for Klentaq and 5-37 °C for Klenow. Both polymerases show significant heat capacity changes upon binding, yielding curved ΔG versus temperature dependences. This results in large changes in ΔH and $T\Delta S$ with temperature, including a sign change from positive to negative for both enthalpy and entropy as temperature increases. For both polymerases, DNA binding is enthalpy-driven near their respective physiological temperatures.

Herein, nucleotide incorporation activity was measured with respect to temperature to examine how the thermodynamics of initial pt-DNA binding relates to the enzymatic activities of Klentaq and Klenow. It is found that both polymerases are enzymatically inactive until the temperature reaches the point where the enthalpy of binding becomes negative (favorable), despite the fact that they both bind DNA quite well at lower temperatures. The data suggest that, for both polymerases, a negative free energy of binding alone is insufficient to drive catalysis, and that a negative enthalpy of initial binding (ΔH) is required for nucleotide incorporation activity. This work is supported by the National Science Foundation.

Membrane Active Peptides II

2482-Pos Board B252

The Clustering of Anionic Lipids by Highly Cationic Cell Penetrating Peptides, as with Antimicrobial Peptides, can Contribute to their Antimicrobial Activity

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It has been reported that there is some overlap of biological activities between cell penetrating peptides (CPPs) and antimicrobial peptides (AMPs). We have studied a group of 9 AMPs, a fusion peptide and 7 CPPs, with regard to their conformational state in the presence and absence of a lipid mixture mimicking the cytoplasmic composition of Gram negative bacteria, their ability to cluster anionic lipids and their bacteriostatic effect on several different species of bacteria. Generally those peptides with the highest number of charges per residue were more effective in clustering anionic lipids. Among the peptides studied, six AMPs and five CPPs were found to have anionic lipid clustering activity. Remarkably, these peptides also had bacteriostatic activity against several species of bacteria, particularly against *E. coli*, a Gram negative bacteria sensitive to lipid clustering agents. In contrast, those AMPs and CPPs that did not cluster anionic lipids were not toxic to *E. coli*. As shown for several types of AMPs previously, we suggest that anionic lipid clustering may contribute to the mechanism of antibacterial action of the more highly cationic CPPs as well. Furthermore, it is a mechanism that should be further considered to explain the entry of these agents into mammalian cells or their escape from intracellular endosomes.

2483-Pos Board B253

Effects of Tryptophan Content and Backbone Spacing on Uptake Efficiency of Cell-Penetrating Peptides

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Cell-penetrating peptides (CPPs) have recently gained considerable interest due to their ability to cross cellular membranes and deliver macromolecular cargo. It is of great importance for the pharmaceutical industry to find molecular tools that can assist drug delivery across the cell plasma membrane and CPPs have emerged as promising candidates for this purpose. Despite being efficient vectors for intracellular delivery, the mechanisms behind CPP uptake into cells are not yet fully understood. Endocytosis has been recognized as a major pathway, however direct translocation pathways also exist and several routes may even act in parallel depending on peptide sequence and concentration.

Our goal is to better understand the mechanistic details of how CPPs enter cells. Previous studies suggest that arginines and tryptophans contribute to cell internalization efficiency. In order to investigate the effect of tryptophan content and backbone spacing on peptide uptake, we have designed a series of peptides all containing eight arginines and 1-4 tryptophan residues with different positions and spacing. Uptake efficiency, cellular distribution and toxicity of these peptides in live mammalian cells were explored using flow cytometry and confocal microscopy. In addition, peptide induced leakage and binding affinity to liposomes were investigated.

Our data show that the uptake efficiency and intracellular distribution varies between peptides of different tryptophan content and backbone spacing. Highest uptake was observed for the peptide with four tryptophans scattered in the amino acid sequence. The peptides were found to have binding constants of the same magnitude, indicating that the differences in uptake are caused by peptide structure rather than variations in binding affinity. All peptides showed relatively low levels of cytotoxicity and liposome leakage, thus making them interesting for future therapeutic applications.

2484-Pos Board B254

Direct Penetration of Cell-Penetrating Peptides Across Lipid Bilayers

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The short, charged, amphipathic and sometimes structured cell-penetrating peptides (CPPs) have been shown promising carriers for delivering protein, RNA, DNA, and even nanoparticles into cells. The mechanistic understanding of how CPPs cross the cell membrane and what biophysical property is critical for efficient penetration is nevertheless largely unknown, mostly due to the lack of proper experimental tools to directly assess the penetration process.

In order to gain insights in the universal rules of penetration mechanism of CPPs, we selected six peptides from three different classes of proposed mechanisms: (1) Tat and poly-arginine as structurally ''disordered'', cationic peptides that most likely enter the cell via endocytosis; (2) penetratin and pVEC as beta-stranded amphipathic peptides shown capable of directly translocate through the membrane; and (3) TP10 and modeled amphipathic peptide (MAP) as alpha-helical amphipathic peptides also being able to penetrate directly. We have developed new techniques based on atomic force microscopy (AFM) to measure energetic and dynamic properties as CPPs go through the bilayer. CPPs were attached via thiol-gold linkage to Au-coated AFM probes and brought into contact with lipid bilayers. The displacement-time trajectory of CPPs inside the bilayers was directly measured during bilayer breakthroughs under different force loading rates. Biophysical characteristics, such as potential energy barrier of bilayer failure, dynamics of penetration pathways, and bilayer structural rearrangement, were derived and compared among the six peptides, which will lead to new classification of CPPs sharing distinguishable biophysical traits.